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Single-Molecule Analysis Suggests Two Novel Pathways for Turning Off Transcription by a MerR-Family Metalloregulator

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Metalloregulators regulate transcription in response to metal ion concentrations. How they interact with DNA and change the DNA structure dictates the regulation process. Many studies have provided insights into how transcription is activated upon metal binding by MerR-family metalloregulators. In contrast, how transcription is turned off after activation is unclear. Turning off transcription promptly is important, however, as the cells would not want to continue expressing metal resistance genes and thus waste energy after metal stress is relieved. Here we use single-molecule fluorescence resonance energy transfer (smFRET) measurements to probe the dynamic interactions between CueR, a Cu1+-responsive MerR-family regulator, and a double-strand DNA in real time one event at a time. Besides seeing its DNA binding and unbinding kinetics, we discovered that CueR spontaneously flips its binding orientation at the recognition site. CueR also has two different binding modes, corresponding to interactions with specific and nonspecific DNA sequences, which would facilitate recognition localization. Most strikingly, a CueR molecule coming from solution can directly substitute for a DNA-bound CueR or assist the dissociation of the incumbent CueR, both of which are the first such examples for any DNA-binding protein. The kinetics of the direct protein substitution and assisted dissociation reactions indicate that these two novel processes can provide efficient pathways to replace a DNA-bound holo-CueR with apo-CueR, thus turning off transcription promptly and facilely.

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Internalization of Fluorescent Biomolecules for Long-Lived Single-Molecule Observation in Living Bacteria

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We present a complete toolbox for the internalization and single-molecule study of singly- or multiply-labeled fluorescent biomolecules (such as DNA and protein) into living E.coli cells. This technique allows use of organic fluorophores, which are much smaller, brighter and more photostable than genetically encoded fluorescent proteins (e.g. GFP), and provide better labeling flexibility (using in vitro site-specific labeling) and wider spectral range. As such, our methods enable experiments that have so far been precluded due to the inability to internalize fairly large molecules such as globular proteins through the cell membranes of micron-size bacterial cells.

Our internalization method, based on electroporation, has allowed us to observe and track fluorescent molecules in living E.coli on the second-to-minute timescale providing >100-times longer observation spans compared to GFP. Aided by the quantized photobleaching of fluorophores, we have characterized the number of internalized molecules, which ranged from 1 to 1000, depending on the size and the amount of electroporated molecule. We also characterized the diffusion behaviour of the internalized molecules, obtaining information on diffusion coefficients, heterogeneity and paths.

By internalising doubly-labeled DNA molecules, we observed single-molecule FRET in single bacteria for the first time. Systematic in vivo single-molecule FRET measurements with DNA standards (exhibiting FRET efficiencies from ~20 to ~90%) show all the hallmarks of single FRET pairs, with efficiencies that agree well with those obtained in vitro. We further demonstrated the technique with labeled proteins, successfully internalizing singly- and doubly-labeled proteins of different sizes. Currently we are extending this method to achieve live-cell super-resolution imaging. Our toolbox should be very helpful for addressing a wide range of questions regarding the structure, interactions and dynamics of bacterial systems in their natural context.

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Probing the Influence of the Particle in Single Particle Tracking Measurements on Lipids

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We present a systematic study of the influence of the nanoparticle used as a probe in Single Particle Tracking experiments with a supported lipid bilayer as a model system. Quantum Dots, 40 nm and 200 nm diameter fluorescent latex spheres, and 40 nm diameter gold colloids were attached to headgroup-labeled lipid molecules in the bilayer by means of an antibody. The percentage of the particle's surface covered with antibody was also varied. We argue that the best estimate of the diffusion coefficient is derived from the first two points of the particle's mean square displacement. The accuracy of this estimate is equivalent to a more rigorous analysis based on the cumulated probability function. We show that, under optimal conditions, Quantum Dots and also 40 nm latex beads have a negligible influence on the lipid diffusion. The diffusion coefficient of gold colloids and 200 nm diameter latex particles was systematically reduced 2- to 3-fold compared to the reference value obtained by Fluorescence Recovery After Photobleaching. Unexpectedly, the diffusion coefficient of Quantum Dots and 40 nm latex beads depended on the percentage of the nanoparticle's surface covered with antibody: at very low percentage the perturbation to the lipid diffusion due to the particle increased. We speculate that in this regime subtle details of antibody binding to the nanoparticle become important.

In an alternative system using particles tethered with 30 or 90 bp DNA strands, particles attached with the longer tether showed the highest diffusion coefficient. We propose that the proximity of the nanoparticle to the membrane may modify lipid dynamics.

Reference

P. Mascalchi *et al*. 2012. Probing the influence of the particle in Single Particle Tracking measurements of lipid diffusion. Soft Matter, 2012,8, 4462-4470.

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A 3-D Image of an Individual Protein

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The dynamic personalities and structural heterogeneity of proteins are essential for proper functioning. Structural determination of dynamic/heterogeneous proteins is limited by conventional approaches of X-ray and electron microscopy (EM) of single-particle reconstruction because they require an average from thousands to millions different molecules. We developed a ''focused electron tomography reconstruction'' (FETR) algorithm to improve the resolution by decreasing the reconstructing image size so that it contains only a single-instance protein. FETR can tolerate certain levels of image-distortion and measuring tilt-errors, and can also precisely determine the translational parameters via an iterative refinement process that contains a series of automatically generated dynamic filters and masks. Since this approach can obtain the structure of a single-instance molecule/particle, we named it individual-particle electron tomography (IPET) as a new robust strategy/approach that does not require a pre-given initial model, class averaging of multiple molecules or an extended ordered lattice, but can tolerate small tilt-errors for high-resolution single ''snapshot'' molecule structure determination. FETR/IPET provides a completely new opportunity for a single-molecule structure determination, and could be used to study the dynamic character and equilibrium fluctuation of macromolecules.

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Unveiling DNA Polymerase Synthesis, Proofreading, and Mutagenesis Activities - One Molecule at a Time

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DNA polymerases must accurately replicate DNA to maintain the integrity of the genome. Carcinogenic adducts, such as 2-aminofluorene (AF) and N-acetyl-2-aminofluorene (AAF), are covalently bound to bases on DNA leading to altered DNA polymerase activity and increased levels of mutagenesis at the adduct site. Although AF and AAF adducts differ only by an acetyl group, the two adducts have different biological effects and the AF adduct is much more easily bypassed by most DNA polymerases. While carcinogen-induced mutagenesis has been extensively studied using ensemble experiments, the exact mechanism for how DNA damage leads to a mutation remains unclear. We have used two complementary single-molecule methods, fluorescence resonance energy transfer (smFRET) and protein-induced fluorescence enhancement (smPIFE), to characterize the interactions between a DNA polymerase and DNA containing an AF or AAF adduct. These studies have allowed us to gain unprecedented insights into the molecular dynamics occurring between a DNA polymerase and a damaged template. We found the degree to which an adduct destabilizes polymerase binding to the DNA depends on the location of the adduct with respect to the primer terminus, the type of adduct, and the nucleotides present in the solution. Not only do the adducts influence the dwell time of the polymerase on the DNA, but also its binding position and orientation. Finally, we have for the first time directly observed the adduct-induced internal transfer of the DNA from the polymerization active site to the 3'-5' exonuclease site.